TRADE SECRET

FINAL REPORT

Study Title
Bacterial Reverse Mutation Assay

Testing Guidelines
U.S. EPA OPPTS Guideline 870.5100 (1998)
EC Commission Directive 2000/32/EC, Annex 4D-B13/14 No. L136
OECD Guidelines for the Testing of Chemicals: Health Effects, No. 471 (1998)

Authors

Study Completion Date
29 November 2010

Testing Facility

Sponsor

Study Number

Sponsor Report Number

Work Request Number

Service Code

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study No. was conducted in compliance with the U.S. EPA GLP Standards 40 CFR 792 and the OECD Principles of Good Laboratory Practice (C(97)186/Final) in all material aspects with the following exceptions:

1. The identity, strength, purity and composition or other characteristics to define the test substance were determined by the Sponsor prior to study initiation. However, the characterization was not conducted in compliance with the above regulations.

   Study Director Impact Statement: The Study Director has concluded that this did not adversely impact the integrity of the data or the validity of the study conclusion since the test substance was characterized prior to use in this study.

2. The stability of the test substance has not been determined by the Testing Facility or the Sponsor.

   Study Director Impact Statement: In the absence of stability data, the Study Director has concluded that the test substance, as supplied, was negative with the caveat that the composition may have changed because of instability.

3. Analyses to determine the uniformity, concentration or stability of the test substance dose formulations were not performed by the testing facility or the Sponsor.

   Study Director Impact Statement: The Study Director has interpretated the data based on the nominal dose levels as documented in the study records and not on the actual formulated test substance concentrations as confirmed by analytical analysis.

Sponsor:

Study Director: 29 Nov 2010
Date

Study Management: 29 Nov 2010
Date

Sponsor: _____ _____
Representative Date

QUALITY ASSURANCE STATEMENT

Quality Assurance Statement

Study Information

Number:

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792
OECD Principles of Good Laboratory Practices (C(97)186/Final)

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
28-Sep-2010	28-Sep-2010	Test System Preparation	28-Sep-2010	28-Sep-2010
28-Sep-2010	29-Sep-2010	Test System Preparation	29-Sep-2010	29-Sep-2010
19-Nov-2010	19-Nov-2010	Data and Draft Reporting	19-Nov-2010	19-Nov-2010
29-Nov-2010	29-Nov-2010	Final Reporting	29-Nov-2010	29-Nov-2010

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

E-signature

Quality Assurance: 29-Nov-2010 6:41 pm GMT

Reason for signature: QA Approval

Printed by

Printed on:29-Nov-10

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director: 29 Nov 2010

Date

Approved by
Sponsor Representative:

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STUDY INFORMATION

Substance Tested:	
Number:	
Composition:	
Purity:	See composition, above
Physical Characteristics:	
Stability:	The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.
Study Initiated/Completed:	September 09, 2010 / (see report cover page)
Experimental Start/Termination:	September 14, 2010 / October 18, 2010

SUMMARY

The test substance, was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA 100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases, using the plate incorporation method. The first phase, the initial toxicity-mutation assay, was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test substance. Dosing formulations were adjusted for purity (20.3%) using a correction factor of 4.93.

Water was used as the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance formed a soluble and clear solution in water at approximately 100 mg/mL, the maximum concentration tested in the solubility test.

In the initial toxicity-mutation assay, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. In the initial toxicity-mutation assay, no positive mutagenic responses were observed. Neither precipitate nor appreciable toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, no positive mutagenic responses were observed with tester strains TA98, TA1535, TA1537 and WP2 *uvr*A in the absence of S9 activation and with any of the tester strains in the presence of S9 activation. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor appreciable toxicity was observed. Due to an unacceptable positive control value, tester strain TA100 in the absence of S9 activation was not evaluated but was retested.

In the retest of the confirmatory mutagenicity assay, the dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. No positive mutagenic response was observed with tester strain TA100 in the absence of S9 activation. Neither precipitate nor appreciable toxicity was observed.

The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, the test substance did not exhibit any mutagenic responses in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test substance was concluded to be negative in this assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9.

A copy of the Historical Negative and Positive Control Values is included in Appendix A. A copy of the study protocol and amendment is included in Appendix B.

The study was conducted to comply with the OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998, OPPTS Guideline 870.5100 (Bacterial reverse mutation test), 1998, and EC Commission Directive 2000/32/EC, Annex 4D-B13/14 No. L136.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, was received by on 23 August 2010 and was assigned the code number Per the protocol, the test substance should be stored at ambient temperature in the dark without desiccant. An expiration date of 01 March 2011 for the test substance was provided on the test article container. Upon receipt, the test substance was described as an amber liquid and was stored at room temperature in the dark without desiccant.

The identity, strength, purity and composition or other characteristics to define the test substance were determined by the Sponsor prior to study initiation. A copy of the Certificate of Analysis was provided to and is maintained in the study records.

The vehicle used to deliver to the test system was water (CAS No.: 7732-18-5, Lot No. 790083, Exp. Date: May 2012), obtained from Invitrogen. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under yellow light. Dosing formulations were adjusted for purity) using a correction factor of

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Positive controls plated concurrently with the mutagenicity assay are listed in the following table. All positive controls were diluted with dimethyl sulfoxide (DMSO) except sodium azide, which was diluted with water. All subdivided solutions of positive control were stored at -15 to -40°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535 and TA1537	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. 03403ED Exp. Date 22-Jan-2012 CAS No. 613-13-8 Purity 99.8%	1.0
TA100			2.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. 03319JD Exp. Date 28-Feb-2011 CAS No. 607-57-8 Purity 98.1%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. 71980 Exp. Date 28-Dec-2010 CAS No. 26628-22-8 Purity 99.8%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. 106F06682 Exp. Date 30-Oct-2010 CAS No. 90-45-9 Purity >97%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. 76296KJ Exp. Date 02-Jun-2012 CAS No. 66-27-3 Purity 99.8%	1,000

To confirm the sterility of the test substance, the highest test substance dose level used in the mutagenicity assay was plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

MATERIALS AND METHODS

Laboratory Manager for this study was Emily W. Dakoulas, BS.

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976). *Salmonella* tester strains were from Dr. Bruce Ames' Master cultures, *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland and both species of tester strain were distributed by MolTox (Boone, NC).

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Overnight cultures were prepared by inoculating from the appropriate master plate, appropriate frozen permanent stock or with a lyophilized pellet into a vessel containing 40 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 to 175 rpm at 37±2°C approximately 12 to 14 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was prepared by and purchased from MolTox (Boone, NC). Upon receipt at the S9 was stored at -60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize at least two promutagens to forms mutagenic to *Salmonella typhimurium* TA 100. The S9 homogenate used in this study was as follows:

Lot	Preparation Date	Protein Content (mg/mL)
2639	29 June 2010	34.0

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM ß- nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Solubility Test

A solubility test was conducted to determine the vehicle. The test was conducted using water up to 100 mg/mL. The test substance formed a soluble and clear solution in water at approximately 100 mg/mL, the maximum concentration tested in the solubility test.

Initial Toxicity-Mutation Test

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. Vehicle control, positive controls and a minimum of eight dose levels of the test substance were plated, two plates per dose, with overnight cultures of TA 98, TA100, TA1535, TA1537 and WP2 *uvr*A on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9.

Confirmatory Mutagenicity Tests

The confirmatory mutagenicity assay was used to evaluate the mutagenic potential of the test substance. A minimum of five dose levels of test substance along with appropriate vehicle control and positive controls were plated with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvr*A on selective agar in the presence and absence of Aroclor-induced rat liver S9. All dose levels of test substance, vehicle control and positive controls were plated in triplicate.

Plating and Scoring Procedures

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D- biotin and L-tryptophan solution to a final concentration of 50 µM each. Top agar not used with S9 or Sham mix was supplemented with 25 mL of water for each 100 mL of minimal top agar. For the preparation of media and reagents, all references to water imply sterile, deionized water. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test substance, test phase, dose level, tester strain and activation, as described in detail in Standard Operating Procedures.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 50 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test substance aliquot was replaced by a 50 µL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over more than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).

Code	Description	Characteristics
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., more than 3 particles on a plate with 30 revertants). These plates are counted manually.

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the assay was the preliminary toxicity assay or the plate exhibited toxicity.

Evaluation of Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance. Data sets for tester strains TA1535 and TA1537 were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value. Data sets for tester strains TA98, TA100 and WP2 *uvr*A were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity assay to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA 98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 0.3×10^9 cells/mL. The mean of at least one positive control per strain and activation condition must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one

or both of the following criteria are met: (1) A > 50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose- dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background lawn code 3, 4 or 5).

Automated Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included but were not limited to the following:

Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments), LIMS System (Excel 2003 (Microsoft Corporation) and Kaye Lab Watch Monitoring System (Kaye GE).

Archives

All raw data, the protocol and all reports, generated by will be maintained according to Standard Operating Procedure by the Quality Assurance unit headquartered at: . Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied onto electronic media and the electronic copy will be retained in the archives for a minimum of 10 years.

Deviations

No known deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Water was used as the solvent of choice based on solubility of the test substance and compatibility with the target cells. The test substance formed a soluble and clear solution in water at approximately 100 mg/mL, the maximum concentration tested in the solubility test.

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions and the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.2	0.3	0.7	1.0	5.6
B2	1.1	0.5	0.7	0.9	3.0
B3	--	0.7	--	--	--

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay are presented in Tables 1 and 2. These data were generated in Experiment B1.

In Experiment B1, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. No positive mutagenic responses were observed. Neither precipitate nor appreciable toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

Confirmatory Mutagenicity Assays

The results of the initial and retest confirmatory mutagenicity assays are presented in Tables 3 through 5. These data were generated in Experiments B2 and B3. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate.

In Experiment B2, no positive mutagenic responses were observed with the tester strains TA98, TA1535, TA1537 and WP2 *uvr*A in the absence of S9 activation and with any of the tester strains in the presence of S9 activation. Neither precipitate nor appreciable toxicity was observed. Due to an

unacceptable positive control value, tester strain TA100 in the absence of S9 activation was not evaluated but was retested.

In Experiment B3, no positive mutagenic response was observed with tester strain TA100 in the absence of S9 activation. Neither precipitate nor appreciable toxicity was observed.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, did not cause a positive response in either the presence or absence of Aroclor-induced rat liver S9. Therefore, the test substance was concluded to be negative in this assay.

Stability data were not provided for the test article. In the absence of stability data, the Study Director has concluded that the test substance, as supplied, was negative with the caveat that the composition may have changed because of instability. Also, at the request of the Sponsor, dosing formulation analysis for concentration and stability was not conducted. Due to the lack of dose formulation analysis, the interpretation of the study data was based on the nominal dose levels as documented in the study records and not on the actual formulated test substance concentrations as confirmed by analytical analysis.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

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Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

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Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

OPPTS Guideline 870.5100 (Bacterial reverse mutation test) (1998).

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

Table 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: Study Code:
Experiment: B1 Date Plated: 9/14/2010
Exposure Method: Plate incorporation assay Evaluation Period: 9/20/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*10*	*2*	*0.6*	11^{A}, 8^{A}
		1500 µg	*8*	*3*	*0.5*	10^{A}, 6^{A}
		500 µg	*12*	*5*	*0.8*	15^{A}, 8^{A}
		150 µg	*12*	*3*	*0.8*	14^{A}, 10^{A}
		50 µg	*12*	*1*	*0.8*	11^{A}, 13^{A}
		15 µg	*13*	*4*	*0.8*	10^{A}, 15^{A}
		5.0 µg	*11*	*3*	*0.7*	9^{A}, 13^{A}
		1.5 µg	*11*	*3*	*0.7*	9^{A}, 13^{A}
	Water	100 µL	*16*	*2*		14^{A}, 17^{A}
TA100		5000 µg	*96*	*4*	*1.0*	93^{A}, 98^{A}
		1500 µg	*93*	*1*	*1.0*	94^{A}, 92^{A}
		500 µg	*115*	*2*	*1.2*	116^{A}, 113^{A}
		150 µg	*92*	*10*	*0.9*	85^{A}, 99^{A}
		50 µg	*88*	*8*	*0.9*	93^{A}, 82^{A}
		15 µg	*87*	*9*	*0.9*	80^{A}, 93^{A}
		5.0 µg	*81*	*3*	*0.8*	79^{A}, 83^{A}
		1.5 µg	*86*	*8*	*0.9*	92^{A}, 80^{A}
	Water	100 µL	*97*	*1*		96^{A}, 97^{A}
TA1535		5000 µg	*13*	*3*	*0.8*	11^{A}, 15^{A}
		1500 µg	*13*	*3*	*0.8*	11^{A}, 15^{A}
		500 µg	*12*	*3*	*0.7*	14^{A}, 10^{A}
		150 µg	*14*	*1*	*0.8*	13^{A}, 15^{A}
		50 µg	*18*	*1*	*1.1*	17^{A}, 19^{A}
		15 µg	*14*	*0*	*0.8*	14^{A}, 14^{A}
		5.0 µg	*14*	*0*	*0.8*	14^{A}, 14^{A}
		1.5 µg	*14*	*1*	*0.8*	15^{A}, 13^{A}
	Water	100 µL	*17*	*5*		13^{A}, 20^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

Continued on next page

Table 1 cont.
Initial Toxicity-Mutation Assay without S9 activation

Study Number: Study Code:
Experiment: B1 Date Plated: 9/14/2010
Exposure Method: Plate incorporation assay Evaluation Period: 9/20/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537		5000 µg	*7*	*2*	*1.8*	8^{A}, 5^{A}
		1500 µg	*4*	*1*	*1.0*	5^{A}, 3^{A}
		500 µg	*7*	*3*	*1.8*	9^{A}, 5^{A}
		150 µg	*4*	*0*	*1.0*	4^{A}, 4^{A}
		50 µg	*6*	*1*	*1.5*	6^{A}, 5^{A}
		15 µg	*8*	*3*	*2.0*	6^{A}, 10^{A}
		5.0 µg	*3*	*3*	*0.8*	5^{A}, 1^{A}
		1.5 µg	*1*	*0*	*0.3*	1^{A}, 1^{A}
	Water	100 µL	*4*	*1*		3^{A}, 5^{A}
WP2uvrA		5000 µg	*52*	*3*	*0.9*	54^{A}, 50^{A}
		1500 µg	*48*	*4*	*0.8*	51^{A}, 45^{A}
		500 µg	*51*	*8*	*0.9*	56^{A}, 45^{A}
		150 µg	*48*	*8*	*0.8*	54^{A}, 42^{A}
		50 µg	*47*	*0*	*0.8*	47^{A}, 47^{A}
		15 µg	*58*	*11*	*1.0*	50^{A}, 65^{A}
		5.0 µg	*50*	*2*	*0.8*	48^{A}, 51^{A}
		1.5 µg	*41*	*6*	*0.7*	45^{A}, 37^{A}
	Water	100 µL	*59*	*0*		59^{A}, 59^{A}
TA98	**2NF**	1.0 µg	*262*	*4*	*16.4*	265^{A}, 259^{A}
TA100	**SA**	1.0 µg	*539*	*16*	*5.6*	550^{A}, 528^{A}
TA1535	**SA**	1.0 µg	*512*	*2*	*30.1*	510^{A}, 513^{A}
TA1537	**9AAD**	75 µg	*748*	*78*	*187.0*	692^{A}, 803^{A}
WP2uvrA	**MMS**	1000 µg	*454*	*8*	*7.7*	448^{A}, 459^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

Table 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: Study Code:
Experiment: B1 Date Plated: 9/14/2010
Exposure Method: Plate incorporation assay Evaluation Period: 9/20/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*27*	*4*	*1.5*	29^{A}, 24^{A}
		1500 µg	*28*	*7*	*1.6*	33^{A}, 23^{A}
		500 µg	*17*	*2*	*0.9*	18^{A}, 15^{A}
		150 µg	*25*	*3*	*1.4*	27^{A}, 23^{A}
		50 µg	*20*	*4*	*1.1*	17^{A}, 23^{A}
		15 µg	*27*	*6*	*1.5*	22^{A}, 31^{A}
		5.0 µg	*23*	*6*	*1.3*	27^{A}, 18^{A}
		1.5 µg	*18*	*4*	*1.0*	20^{A}, 15^{A}
	Water	100 µL	*18*	*1*		19^{A}, 17^{A}
TA100		5000 µg	*100*	*15*	*1.0*	110^{A}, 89^{A}
		1500 µg	*94*	*6*	*0.9*	89^{A}, 98^{A}
		500 µg	*93*	*12*	*0.9*	101^{A}, 84^{A}
		150 µg	*95*	*10*	*0.9*	102^{A}, 88^{A}
		50 µg	*111*	*6*	*1.1*	115^{A}, 107^{A}
		15 µg	*97*	*13*	*0.9*	106^{A}, 87^{A}
		5.0 µg	*106*	*7*	*1.0*	101^{A}, 111^{A}
		1.5 µg	*94*	*6*	*0.9*	98^{A}, 89^{A}
	Water	100 µL	*105*	*20*		91^{A}, 119^{A}
TA1535		5000 µg	*8*	*0*	*1.1*	8^{A}, 8^{A}
		1500 µg	*7*	*2*	*1.0*	5^{A}, 8^{A}
		500 µg	*10*	*6*	*1.4*	14^{A}, 6^{A}
		150 µg	*7*	*3*	*1.0*	5^{A}, 9^{A}
		50 µg	*12*	*2*	*1.7*	13^{A}, 10^{A}
		15 µg	*7*	*2*	*1.0*	5^{A}, 8^{A}
		5.0 µg	*9*	*1*	*1.3*	8^{A}, 9^{A}
		1.5 µg	*8*	*4*	*1.1*	10^{A}, 5^{A}
	Water	100 µL	*7*	*4*		4^{A}, 9^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

Continued on next page

Table 2 cont.
Initial Toxicity-Mutation Assay with S9 activation

Study Number: Study Code:
Experiment: B1 Date Plated: 9/14/2010
Exposure Method: Plate incorporation assay Evaluation Period: 9/20/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA1537		5000 µg	*10*	*5*	*1.4*	6^{A}, 13^{A}
		1500 µg	*5*	*0*	*0.7*	5^{A}, 5^{A}
		500 µg	*6*	*3*	*0.9*	8^{A}, 4^{A}
		150 µg	*7*	*2*	*1.0*	8^{A}, 5^{A}
		50 µg	*8*	*4*	*1.1*	5^{A}, 10^{A}
		15 µg	*7*	*2*	*1.0*	5^{A}, 8^{A}
		5.0 µg	*6*	*0*	*0.9*	6^{A}, 6^{A}
		1.5 µg	*5*	*1*	*0.7*	4^{A}, 5^{A}
	Water	100 µL	*7*	*3*		9^{A}, 5^{A}
WP2uvrA		5000 µg	*49*	*4*	*1.0*	51^{A}, 46^{A}
		1500 µg	*35*	*11*	*0.7*	27^{A}, 42^{A}
		500 µg	*51*	*5*	*1.0*	47^{A}, 54^{A}
		150 µg	*47*	*1*	*0.9*	48^{A}, 46^{A}
		50 µg	*65*	*6*	*1.3*	69^{A}, 60^{A}
		15 µg	*46*	*1*	*0.9*	45^{A}, 47^{A}
		5.0 µg	*42*	*5*	*0.8*	38^{A}, 45^{A}
		1.5 µg	*40*	*5*	*0.8*	36^{A}, 43^{A}
	Water	100 µL	*51*	*4*		54^{A}, 48^{A}
TA98	**2AA**	1.0 µg	*341*	*52*	*18.9*	377^{A}, 304^{A}
TA100	**2AA**	2.0 µg	*739*	*115*	*7.0*	658^{A}, 820^{A}
TA1535	**2AA**	1.0 µg	*118*	*8*	*16.9*	112^{A}, 124^{A}
TA1537	**2AA**	1.0 µg	*136*	*4*	*19.4*	139^{A}, 133^{A}
WP2uvrA	**2AA**	10 µg	*272*	*24*	*5.3*	289^{A}, 255^{A}

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

Table 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: Study Code:
Experiment: B2 Date Plated: 9/28/2010
Exposure Method: Plate incorporation assay Evaluation Period: 10/4/2010 to 10/5/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*9*	*1*	*0.6*	8^{A}, 8^{A}, 10^{A}
		1500 µg	*17*	*2*	*1.1*	15^{A}, 17^{A}, 18^{A}
		500 µg	*10*	*1*	*0.6*	11^{A}, 9^{A}, 9^{A}
		150 µg	*15*	*6*	*0.9*	22^{A}, 14^{A}, 10^{A}
		50 µg	*15*	*2*	*0.9*	14^{A}, 17^{A}, 14^{A}
	Water	100 µL	*16*	*2*		17^{A}, 14^{A}, 17^{A}
TA1535		5000 µg	*12*	*2*	*2.0*	13^{A}, 10^{A}, 13^{A}
		1500 µg	*7*	*7*	*1.2*	1^{A}, 6^{A}, 14^{A}
		500 µg	*7*	*1*	*1.2*	6^{A}, 8^{A}, 8^{A}
		150 µg	*7*	*5*	*1.2*	1^{A}, 9^{A}, 11^{A}
		50 µg	*6*	*2*	*1.0*	6^{A}, 8^{A}, 4^{A}
	Water	100 µL	*6*	*2*		5^{A}, 5^{A}, 9^{A}
TA1537		5000 µg	*5*	*1*	*1.3*	6^{A}, 4^{A}, 6^{A}
		1500 µg	*4*	*0*	*1.0*	4^{A}, 4^{A}, 4^{A}
		500 µg	*4*	*1*	*1.0*	3^{A}, 4^{A}, 5^{A}
		150 µg	*6*	*5*	*1.5*	11^{A}, 5^{A}, 1^{A}
		50 µg	*5*	*1*	*1.3*	6^{A}, 6^{A}, 4^{A}
	Water	100 µL	*4*	*2*		4^{A}, 3^{A}, 6^{A}
WP2uvrA		5000 µg	*36*	*3*	*1.0*	37^{A}, 33^{A}, 38^{A}
		1500 µg	*34*	*7*	*1.0*	36^{A}, 27^{A}, 40^{A}
		500 µg	*35*	*6*	*1.0*	31^{A}, 42^{A}, 32^{A}
		150 µg	*32*	*6*	*0.9*	29^{A}, 28^{A}, 38^{A}
		50 µg	*27*	*6*	*0.8*	22^{A}, 34^{A}, 26^{A}
	Water	100 µL	*35*	*11*		29^{A}, 47^{A}, 28^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

Continued on next page

Table 3 cont.
Confirmatory Mutagenicity Assay without S9 activation

Study Number: Study Code:
Experiment: B2 Date Plated: 9/28/2010
Exposure Method: Plate incorporation assay Evaluation Period: 10/4/2010 to 10/5/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2NF**	1.0 µg	*308*	*57*	*19.3*	355[A], 324[A], 244[A]
TA1535	**SA**	1.0 µg	*177*	*20*	*29.5*	191[A], 185[A], 154[A]
TA1537	**9AAD**	75 µg	*427*	*198*	*106.8*	589[A], 486[A], 207[A]
WP2uvrA	**MMS**	1000 µg	*417*	*25*	*11.9*	446[A], 400[A], 406[A]

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic & Manual Count Flags

[M]: Manual count [A]: Automatic count

Table 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: Study Code:
Experiment: B2 Date Plated: 9/28/2010
Exposure Method: Plate incorporation assay Evaluation Period: 10/4/2010 to 10/5/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98		5000 µg	*17*	*2*	*0.9*	18^{A}, 18^{A}, 14^{A}
		1500 µg	*24*	*5*	*1.3*	29^{A}, 20^{A}, 24^{A}
		500 µg	*21*	*4*	*1.1*	18^{A}, 26^{A}, 20^{A}
		150 µg	*17*	*4*	*0.9*	14^{A}, 15^{A}, 22^{A}
		50 µg	*24*	*7*	*1.3*	17^{A}, 24^{A}, 31^{A}
	Water	100 µL	*19*	*5*		19^{A}, 24^{A}, 15^{A}
TA100		5000 µg	*149*	*17*	*1.0*	145^{A}, 134^{A}, 168^{A}
		1500 µg	*140*	*9*	*1.0*	145^{A}, 145^{A}, 130^{A}
		500 µg	*148*	*26*	*1.0*	140^{A}, 177^{A}, 128^{A}
		150 µg	*145*	*6*	*1.0*	140^{A}, 144^{A}, 152^{A}
		50 µg	*139*	*8*	*1.0*	144^{A}, 130^{A}, 144^{A}
	Water	100 µL	*143*	*19*		165^{A}, 131^{A}, 133^{A}
TA1535		5000 µg	*15*	*2*	*1.7*	17^{A}, 14^{A}, 15^{A}
		1500 µg	*12*	*5*	*1.3*	9^{A}, 10^{A}, 18^{A}
		500 µg	*10*	*2*	*1.1*	11^{A}, 10^{A}, 8^{A}
		150 µg	*12*	*1*	*1.3*	13^{A}, 11^{A}, 13^{A}
		50 µg	*14*	*6*	*1.6*	13^{A}, 9^{A}, 20^{A}
	Water	100 µL	*9*	*4*		10^{A}, 13^{A}, 5^{A}
TA1537		5000 µg	*6*	*2*	*0.8*	6^{A}, 4^{A}, 8^{A}
		1500 µg	*6*	*3*	*0.8*	9^{A}, 4^{A}, 4^{A}
		500 µg	*6*	*3*	*0.8*	4^{A}, 9^{A}, 5^{A}
		150 µg	*4*	*1*	*0.5*	4^{A}, 4^{A}, 3^{A}
		50 µg	*8*	*4*	*1.0*	3^{A}, 11^{A}, 10^{A}
	Water	100 µL	*8*	*4*		4^{A}, 10^{A}, 11^{A}
WP2uvrA		5000 µg	*35*	*5*	*0.8*	41^{A}, 32^{A}, 33^{A}
		1500 µg	*35*	*6*	*0.8*	41^{A}, 32^{A}, 31^{A}
		500 µg	*44*	*9*	*1.0*	48^{A}, 34^{A}, 51^{A}
		150 µg	*39*	*6*	*0.9*	43^{A}, 42^{A}, 33^{A}
		50 µg	*34*	*5*	*0.8*	28^{A}, 36^{A}, 37^{A}
	Water	100 µL	*44*	*3*		47^{A}, 45^{A}, 41^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

Continued on next page

Table 4 cont.
Confirmatory Mutagenicity Assay with S9 activation

Study Number: Study Code:
Experiment: B2 Date Plated: 9/28/2010
Exposure Method: Plate incorporation assay Evaluation Period: 10/4/2010 to 10/5/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2AA**	1.0 µg	*265*	*24*	*13.9*	287[A], 269[A], 240[A]
TA100	**2AA**	2.0 µg	*609*	*159*	*4.3*	793[A], 519[A], 516[A]
TA1535	**2AA**	1.0 µg	*127*	*33*	*14.1*	156[A], 134[A], 91[A]
TA1537	**2AA**	1.0 µg	*74*	*9*	*9.3*	75[A], 82[A], 64[A]
WP2uvrA	**2AA**	10 µg	*214*	*14*	*4.9*	198[A], 219[A], 224[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic & Manual Count Flags

[M]: Manual count [A]: Automatic count

Table 5
Retest of the Confirmatory Mutagenicity Assay without S9 activation

Study Number: Study Code:
Experiment: B3 Date Plated: 10/12/2010
Exposure Method: Plate incorporation assay Evaluation Period: 10/18/2010

Strain	Article	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA100		5000 µg	*92*	*12*	*1.1*	89[A], 105[A], 81[A]
		1500 µg	*78*	*11*	*0.9*	82[A], 65[A], 86[A]
		500 µg	*92*	*9*	*1.1*	97[A], 97[A], 82[A]
		150 µg	*82*	*11*	*1.0*	70[A], 92[A], 84[A]
		50 µg	*79*	*3*	*1.0*	76[A], 82[A], 78[A]
	Water	100 µL	*83*	*7*		81[A], 90[A], 77[A]
TA100	**SA**	1.0 µg	*529*	*46*	*6.4*	550[A], 560[A], 476[A]

Key to Positive Controls

SA sodium azide

Key to Automatic & Manual Count Flags

[M]: Manual count [A]: Automatic count

APPENDIX A: Historical Control Data

Historical Negative and Positive Control Values
2007 – 2009

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	18	7	4	57	25	8	6	69
	Pos	221	131	34	1299	526	245	49	2342
TA100	Neg	132	32	51	255	141	35	56	268
	Pos	613	153	226	1837	776	380	224	3206
TA1535	Neg	17	7	3	58	15	5	1	49
	Pos	456	159	33	1601	120	85	18	1216
TA1537	Neg	8	4	0	28	8	4	1	41
	Pos	1040	576	24	4814	102	156	13	2360
WP2 *uvr*A	Neg	28	11	6	72	31	12	5	77
	Pos	344	163	44	1178	274	131	32	1656

SD=standard deviation; Min= minimum value; Max=maximum value; Neg=ne gative control (including but not li mited to deionized water, di methyl sulfoxi de, ethanol and ac etone); Pos=positive control

APPENDIX B: Study Protocol

PROTOCOL AMENDMENT 1

Sponsor:

Study No.: **Work Request No.:**
Number: **Sponsor Report No.:**

Title: Bacterial Reverse Mutation Assay

1. Page 3, §4.4 Quality Assurance Unit of Lead QA):

   Change the QA lead to:

   QA Reviewed

   Quality Specialist Supervisor, Genetic Toxicology
   Phone:
   Fax:
   Email:

   PKB 24NOV2010
   Init. Date

   Reason: responsibilities have changed, and he is no longer the Lead QA contact person.

Approvals:

22 Nov 2010
Date

22 NOV 2010
Date

24 NOV 2010
Date

QA Reviewed
AM 16Sep10
Init. Date

Received by RA/QA 10-Sep-2010

Study Number:

Bacterial Reverse Mutation Assay

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of S9 activation.

2.0 SPONSOR

2.1 Sponsor/ Applicant Name:

2.2 Representative:

2.3 Work Request No.:

2.4 Number:

2.5 Service Code:

2.6 Sponsor Report No.:

3.0 TEST AND CONTROL SUBSTANCES

3.1 Test Substance Name:

Name to be used in report title:

Name to be used in report text:

Storage Temperature: Ambient

Study Number:

Storage Parameters: The test substance will be stored in the dark without desiccant.

Purity: **The dosing preparations will be adjusted for purity using a correction factor of**

3.2 Controls: Negative: Test substance vehicle
Positive: 9-aminoacridine
2-aminoanthracene
methyl methanesulfonate
2-nitrofluorene
sodium azide

3.3 Characterization and Stability of the Test Substance

will not perform analysis of the test substance. The Sponsor will be directly responsible for determination and documentation of the analytical purity, composition and stability of the test substance, and the stability and strength of the test substance in the solvent (or vehicle). If there is no characterization and/or stability analysis of the test substance, the GLP compliance statement in the final report will cite these deficiencies as exceptions to the GLP regulations with which this study is compliant. If there is no characterization and/or stability analysis of the test substance formulation, the GLP compliance statement in the final report will cite these deficiencies as exceptions to the GLP regulations with which this study is compliant.

3.4 Test Substance Retention Sample

Since the in-life portion of this study is less than four weeks in duration, will not retain a reserve sample of the test substance.

3.5 Residual Test Substance and Dosing Preparations

Dosing preparations, excluding those saved for concentration or homogeneity analysis (if any), will be disposed of following administration to the test system. Following finalization of the report, residual test substance will be discarded unless otherwise indicated by the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name:

4.2 Address:

Study Number:

4.3 Study Director:

4.4 Quality Assurance Unit of (Lead QA):

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 14 Sep 2010

5.2 Proposed Experimental Completion Date: 19 Oct 2010

5.3 Proposed Report Date: 02 Nov 2010

6.0 TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976).

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

Each *S. typhimurium* tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The *rfa* mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the *uvr*B gene results in a deficient DNA excision-repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both

frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The *E. coli* tester strain has an AT base pair at the critical mutation site within the *trp*E gene (Wilcox *et al.*, 1990). Tester strain WP2 *uvr*A has a deletion in the *uvr*A gene resulting in a deficient DNA excision-repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations (Green and Muriel, 1976).

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox) using cultures derived from the above sources.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

7.1 Solubility Determination

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice will be the solvent, selected in order of preference, which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the solvents to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

7.2 Initial Toxicity-Mutation Assay

Selection of dose levels for the confirmatory mutagenicity assay will be based upon the toxicity and precipitation profile of the test substance assessed in an initial toxicity-mutation assay. The test substance will be tested at a minimum of eight dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvr*A with and without S9 activation. All dose levels of test substance, negative controls and positive controls will be plated in duplicate. Unless indicated otherwise by the Sponsor, the highest dose will be the highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. Solubility or workability permitting, the dose levels will be 5000, 1500, 500, 150, 50, 15, 5.0 and 1.5 µg per plate. In selecting dose levels for the confirmatory mutagenicity assay the following guidelines will be employed. Doses will be selected such that precipitate does not interfere with manual scoring.

Whenever possible, the highest dose for the confirmatory mutagenicity assay will be selected to give some indication of toxicity without exceeding 5 mg/plate. For freely soluble, nontoxic test substances, the highest dose level will be 5 mg/plate. For precipitating, nontoxic test substances, the highest dose level may be selected in an attempt to yield precipitate at only the top one or two dose levels. The Sponsor will be consulted regarding dose selection if (1) the maximum dose level is selected based on precipitation and this dose level is less than 5 mg/plate or (2) the maximum achievable test substance dose level is less than 5 mg/plate and this dose level is nontoxic. The doses selected for the confirmatory mutagenicity assay will be documented in the raw data and report. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

7.3 Confirmatory Mutagenicity Assay

The test substance will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvr*A with and without S9 activation. All dose levels of test substance, negative controls and positive controls will be plated in triplicate.

7.4 Frequency and Route of Administration

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay. Verification of a clear positive response is not required. Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method). This guidance is based on the OECD Guideline 471 (1998) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1997).

7.5 Controls

No analyses will be performed on the positive control substances or the positive control dose formulations. The neat positive control substances and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at

Study Number:

7.5.1 Positive Controls

The positive controls that will be plated concurrently with the assay are listed below. Results obtained from these substances will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	S9	Positive Control	Concentration (µg/plate)
Salmonella Strains	Rat	2-aminoanthracene	1.0-2.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene	1.0
TA100, TA1535		sodium azide	1.0
TA1537		9-aminoacridine	75
WP2 *uvr*A		methyl methanesulfonate	1,000

7.5.2 Negative Controls

Appropriate negative controls will be plated for each tester strain with and without S9 activation. The negative control will be the vehicle alone, unless there is no historical basis for use of the selected vehicle. In the latter case, both untreated and vehicle controls will be used.

7.5.3 Sterility Controls

The most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

7.6 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 homogenate was or will be purchased from Moltox and stored frozen at -60°C or colder until used. Each batch of S9 homogenate was or will be assayed for its ability to metabolize at least two promutagens to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

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7.7 Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate master plate, from the appropriate frozen stock or with the appropriate lyophilized pellet(s). To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a shaker/incubator programmed to begin shaking at approximately 125 to 175 rpm and incubating at 37±2°C for approximately 12 to 14 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.8 Test System Identification

Each plate will be labeled with a code system that identifies the test substance, test phase, dose level, tester strain and activation type as described in Standard Operating Procedures.

7.9 Test Substance Preparation

Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under yellow light.

7.10 Treatment of Test System

One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.11 Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the negative control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn.

Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.12 Tester Strain Verification

On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers cited in §6.0.

7.13 Automated Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis may include but not limited to the following:

Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments), LIMS System (BioReliance), Excel 2003 (Microsoft Corporation) and Kaye Lab Watch Monitoring System (Kaye GE).

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Negative Controls Values

Based on historical control data, all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. Untreated controls, when part of the design, must also be within the ranges cited above.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than $0.3x10^9$ cells per milliliter.

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8.4 Positive Control Values

Each mean, positive control value must exhibit at least a 3.0-fold increase over the respective mean, negative control value (vehicle) for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

9.1 Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value.

9.2 Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. Unless alternate arrangements are made, the report will be initially issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. The report will include:

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- Test Substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, if any; statistical analysis, if any; concurrent negative and positive control data means and standard deviations.
- Discussion of results
- Conclusion
- Appendices: Historical Control Data (negative and positive controls with ranges, means and standard deviations), copy of protocol and any amendment, and, if provided by the Sponsor, copies of the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations.
- Statement of Compliance
- Quality Assurance Statement

If an electronic copy of the protocol, the report or another study document is provided by the executed paper document is considered the official master document. If there is a discrepancy between an electronic copy and the corresponding master document, the master document will be considered the official document. Six months after issuance of the draft report, if no requested revisions or instructions to finalize have been communicated by the Sponsor or a designated representative, the draft report will be issued as a final report. If all supporting analytical documents have not been provided to , the report will be written based on those that are provided to

11.0 RECORDS AND ARCHIVES

All raw data, the protocol and all reports, generated by will be maintained according to Standard Operating Procedure by the Quality Assurance unit headquartered at:

Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied onto electronic media and the electronic copy will be retained in the archives for a minimum of 10 years.

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12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998, OPPTS Guideline 870.5100 (Bacterial reverse mutation test), 1998, EC Commission Directive 2000/32/EC, Annex 4D-B13/14 No. L136.

The following Good Laboratory Practices (GLP) regulations will be followed at as requested by the Sponsor.

- OECD Principles of Good Laboratory Practice (C(97)186/Final)
- US EPA GLP Standards 40 CFR 792

For the study, an in-process phase, the raw data, and report(s) will be inspected per the Standard Operating Procedures (SOPs) of by the Quality Assurance Unit of for compliance with GLPs, the SOPs of and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA Statement will be included in the final report. This statement will list the study-specific phases inspected at the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP Compliance Statement will be included in the final report. This statement will cite the GLP regulations with which this study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test substance or its mixtures.

Raw data, the protocol and reports generated at locations other than will or will not be QA audited per the contractual arrangements between that site and the Sponsor.

Alterations of this protocol may be made as the study progresses. All protocol procedural modifications and rationale for the change(s) will be documented, signed, dated and approved by the Study Director, Study Management and the Sponsor. QA will review all protocol amendments and document this review by initials and date. All applicable protocol amendments will be delivered by physical or electronic means to the Sponsor representative, within the Test Facility, and if applicable, to the test site(s) and Study Monitor(s).

Deviations from the protocol and/or SOPs will be documented in a deviation report or a note to file will be generated. The deviation report will be signed by the Study Director and QA.

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13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

EC Commission Directive 2000/32/EC, Annex 4D-B13/14 No. L136.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

OPPTS Guideline 870.5100 (Bacterial reverse mutation test), 1998.

Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains. Mutagenesis 5:285-291.

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14.0 APPROVALS

14.1 Sponsor Approval

25-AUG-2010
Date

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14.2 Study Director and Test Facility Management Approvals

09 Sep 2010
Date

10Sep2010
Date